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METHOD OF DIAGNOSING HEPTOCELLULAR CARCINOMAS

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Respectfully submitted,

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DESCRIPTION

METHOD FOR DIAGNOSING HEPATOCELLULAR CARCINOMAS

FIELD OF THE INVENTION

The invention relates to methods of diagnosing hepatocellular carcinomas.

BACKGROUND OF THE INVENTION

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. In spite of recent progress in diagnostic and therapeutic strategies, prognosis of patients with advanced cancer remains very poor. Although molecular studies have revealed that alteration of tumor suppressor genes and/or oncogenes is involved in their carcinogenesis, the precise mechanisms remain to be fully elucidated. To disclose mechanisms underlying this tumor from a genome-wide point of view and to discover target molecules for diagnosis and for development of novel therapeutic drugs, we have been analyzing the expression profile by means of a cDNA microarray representing 23,040 genes (1, 2, 3). The effort have identified a number of genes, including ESTs, which appear to be up-regulated frequently in the cancer tissues compared with the corresponding non-cancerous cells. Since carcinogenesis involves activation of oncogenes and/or inactivation of tumor suppressor genes, enhanced expression of at least some of these up-regulated genes may reflect oncogenic properties.

cDNA microarray technologies have enabled to obtain comprehensive profiles of gene expression in normal and malignant cells, and compare the gene expression in malignant and corresponding normal cells (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61: 3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)). This approach enables to disclose the complex nature of cancer cells, and helps to understand the mechanism of carcinogenesis. Identification of genes that are deregulated in tumors can lead to more precise and accurate diagnosis of individual cancers, and to develop novel therapeutic targets (Bienz and Clevers, Cell 103:311-20 (2000)). To disclose mechanisms underlying tumors from a genome-wide point of view, and discover target molecules for diagnosis and development of novel therapeutic drugs, the present inventors have been analyzing the expression profiles of tumor cells using a cDNA microarray of 23040 genes (Okabe et al., Cancer Res 61:2129-37 (2001); Kitahara et al., Cancer Res 61:3544-9 (2001); Lin et al., Oncogene 21:4120-8 (2002); Hasegawa et al., Cancer Res 62:7012-7 (2002)).

Studies designed to reveal mechanisms of carcinogenesis have already facilitated identification of molecular targets for anti-tumor agents. For example, inhibitors of farnexyltransferase (FTIs) which were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends on posttranslational farnesylation, has been effective in treating Ras-dependent tumors in animal models (He et al., Cell 99:335-45 (1999)). Clinical trials on human using a combination or anti-cancer drugs and anti-HER2 monoclonal antibody, trastuzumab, have been conducted to antagonize the proto-oncogene receptor HER2/neu; and have been achieving improved clinical response and overall survival of breast-cancer patients (Lin et al., Cancer Res 61:6345-9 (2001)). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcr-abl fusion proteins, has been developed to treat chronic myelogenous leukemias wherein constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes. Agents of these kinds are designed to suppress oncogenic activity of specific gene products (Fujita et al., Cancer Res 61:7722-6 (2001)). Therefore, gene products commonly up-regulated in cancerous cells may serve as potential targets for developing novel anti-cancer agents.

It has been demonstrated that CD8+ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecule, and lyse tumor cells. Since the discovery of MAGE family as the first example of TAAs, many other TAAs have been discovered using immunological approaches (Boon, Int J Cancer 54: 177-80 (1993); Boon and van der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al., J Exp Med 180: 347-52 (1994)). Some of the discovered TAAs are now in the stage of clinical development as targets of immunotherapy. TAAs discovered so far include MAGE (van der Bruggen et al., Science 254: 1643-7 (1991)), gp100 (Kawakami et al., J Exp Med 180: 347-52 (1994)), SART (Shichijo et al., J Exp Med 187: 277-88 (1998)), and NY-ESO-1 (Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997)). On the other hand, gene products which had been demonstrated to be specifically overexpressed in tumor cells, have been shown to be recognized as targets inducing cellular immune responses. Such gene products include p53 (Umano et al., Brit J Cancer 84: 1052-7 (2001)), HER2/neu (Tanaka et al., Brit J Cancer 84: 94-9 (2001)), CEA (Nukaya et al., Int J Cancer 80: 92-7 (1999)), and so on.

In spite of significant progress in basic and clinical research concerning TAAs (Rosenbeg et al., Nature Med 4: 321-7 (1998); Mukherji et al., Proc Natl Acad Sci USA 92: 8078-82 (1995); Hu et al., Cancer Res 56: 2479-83 (1996)), only limited number of candidate TAAs for the

treatment of adenocarcinomas, including hepatocellular carcinoma, are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells would be promising candidates as immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and can der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al.,J Exp Med 180: 347-52 (1994); Shichijo et al., J Exp Med 187: 277-88 (1998); Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997); Harris, J Natl Cancer Inst 88: 1442-5 (1996); Butterfield et al., Cancer Res 59: 3134-42 (1999); Vissers et al., Cancer Res 59: 5554-9 (1999); van der Burg et al., J Immunol 156: 3308-14 (1996); Tanaka et al., Cancer Res 57: 4465-8 (1997); Fujie et al., Int J Cancer 80: 169-72 (1999); Kikuchi et al., Int J Cancer 81: 459-66 (1999); Oiso et al., Int J Cancer 81: 387-94 (1999)).

It has been repeatedly reported that peptide-stimulated peripheral blood mononuclear cells (PBMCs) from certain healthy donors produce significant levels of IFN-γ in response to the peptide, but rarely exert cytotoxicity against tumor cells in an HLA-A24 or –A0201 restricted manner in ^{51}Cr-release assays (Kawano et al., Cance Res 60: 3550-8 (2000); Nishizaka et al., Cancer Res 60: 4830-7 (2000); Tamura et al., Jpn J Cancer Res 92: 762-7 (2001)). However, both of HLA-A24 and HLA-A0201 are one of the popular HLA alleles in Japanese, as well as Caucasian (Date et al., Tissue Antigens 47: 93-101 (1996); Kondo et al., J Immunol 155: 4307-12 (1995); Kubo et al., J Immunol 152: 3913-24 (1994); Imanishi et al., Proceeding of the eleventh International Hictocompatibility Workshop and Conference Oxford University Press, Oxford, 1065 (1992); Williams et al., Tissue Antigen 49: 129 (1997)). Thus, antigenic peptides of carcinomas presented by these HLAs may be especially useful for the treatment of carcinomas among Japanese and Caucasian. Further, it is known that the induction of low-affinity CTL *in vitro* usually results from the use of peptide at a high concentration, generating a high level of specific peptide/MHC complexes on antigen presenting cells (APCs), which will effectively activate these CTL (Alexander-Miller et al., Proc Natl Acad Sci USA 93: 4102-7 (1996)).

SUMMARY OF THE INVENTION

To disclose mechanisms of hepatocellular carcinogenesis and identify novel diagnostic markers and molecular targets of anticancer drugs for hepatocellular carcinoma (HCC), we analyzed expression profiles of 20 HCCs using a genome-wide cDNA microarray containing

23040 genes. Among the genes with altered expression in the tumors, we focused on two novel human gene, *MGC47816* and *HES6*, frequently up-regulated in the cancers compared with the corresponding normal tissues. One gene, *MGC47816*, encoded a putative 391-amino-acid protein containing a carbamoyl-phosphate synthase L chain and an ATP binding domain, and was assigned at chromosomal band 1q34.1. Another gene, *HES6*, suppressed expression of *MGC47816* by transfection of short interfering RNA (siRNA) inhibited the growth of hepatocellular carcinoma cells. These results provide novel insight into hepatocellular carcinogenesis, and may contribute to the development of new strategies for diagnosis and treatment of this cancer.

The invention is based on the discovery of a pattern of gene expression of MGC47816 and HES6 correlated with hepatocellular carcinoma (HCC).

Accordingly, the invention features a method of diagnosing or determining a predisposition to HCC in a subject by determining an expression level of MGC47816 or HES6 in a patient derived biological sample, such as tissue sample. A normal cell is one obtained from hepatocellular tissue. An increase of the level of expression of the MGC47816 or HES6 compared to a normal control level of the gene indicates that the subject suffers from or is at risk of developing HCC.

By normal control level is meant a level of gene expression detected in a normal, healthy individual or in a population of individuals known not to be suffering from HCC. A control level is a single expression pattern derived from a single reference population or from a plurality of expression patterns. For example, the control level can be a database of expression patterns from previously tested cells. A normal individual is one with no clinical symptoms of HCC.

An increase in the level of expression of MGC47816 or HES6 detected in a test sample compared to a normal control level indicates the subject (from which the sample was obtained) suffers from or is at risk of developing HCC.

Gene expression is increased 10%, 25%, 50% compared to the control level. Alternately, gene expression is increased 0.1, 0.2, 1, 2, 5, 10 or more fold compared to the control level. Expression is determined by detecting hybridization, *e.g.*, MGC47816 and HES6 gene probe to a gene transcript of the patient-derived tissue sample.

The patient derived tissue sample is any tissue from a test subject, *e.g.*, a patient known to or suspected of having HCC. For example, the tissue contains a liver cancer cell. For example, the tissue is a cell from liver.

Hepatocellular Carcinoma

The invention further provides methods of identifying an agent that inhibits the expression or activity of MGC47816 or HES6 by contacting a test cell expressing MGC47816 or HES6 with a test agent and determining the expression level or activity of the MGC47816 or HES6. The test cell is a hepatocellular cell such as a hepatocellular cell from a hepatocellular carcinoma. A decrease of the level compared to a normal control level of the gene indicates that the test agent is an inhibitor of the MGC47816 or HES6 and reduces a symptom of HCC.

The invention also provides a kit with a detection reagent which binds to MGC47816 or HES6 nucleic acid sequences or which binds to a gene product encoded by the nucleic acid sequences.

Therapeutic methods include a method of treating or preventing HCC in a subject by administering to the subject an antisense composition. The antisense composition reduces the expression of a specific target gene, *e.g.*, the antisense composition contains a nucleotide, which is complementary to a nucleic acid sequence of MGC47816 or HES6. Another method includes the steps of administering to a subject an small interfering RNA (siRNA) composition. The siRNA composition reduces the expression of a nucleic acid of MGC47816 or HES6. In yet another method, treatment or prevention of HCC in a subject is carried out by administering to a subject a ribozyme composition. The nucleic acid-specific ribozyme composition reduces the expression of a nucleic acid of MGC47816 or HES6.Suitable mechanisms for *in vivo* expression of a gene of interest are known in the art.

The invention also includes vaccines and vaccination methods. For example, a method of treating or preventing HCC in a subject is carried out by administering to the subject a vaccine containing a polypeptide encoded by a nucleic acid of MGC47816 or HES6 or an immunologically active fragment such a polypeptide. An immunologically active fragment is a polypeptide that is shorter in length than the full-length naturally-occurring protein and which induces an immune response. For example, an immunologically active fragment at least 8 residues in length and stimulates an immune cell such as a T cell or a B cell. Immune cell stimulation is measured by detecting cell proliferation, elaboration of cytokines (*e.g.*, IL-2), or production of an antibody.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned

herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

One advantage of the methods described herein is that the disease is identified prior to detection of overt clinical symptoms. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts relative expression ratios (cancer/non-cancer) of D4999 in primary 20 HCCs examined by cDNA microarray. Its up-regulated expression (Cy3:Cy5 intensity ratio, >2.0) was observed in 7 of the 11 HCCs that passed through the cutoff filter (both Cy3 and Cy5 signals greater than 25,000).

Figure 2 depicts expression of D4999 analyzed by semi-quantitative RT-PCR using additional HCC tissues. T, tumor tissue; N, normal tissue. Expression of *GAPDH* served as an internal control.

Figure 3 depicts genomic structure of *MGC47816* and structure of the predicted MGC47816 protein. Exons are indicated by open boxes with nucleotide numbers of *MGC47816* cDNA in the upper panel.

Figure 4 depicts subcellular localization of HA-tagged MGC47816 protein. Immunoblotting of HA-tagged MGC47816 protein (a). Immunohistochemical staining of the tagged proteins in COS7 cells (b). The protein was stained with rat anti-HA monoclonal antibody and visualized by RHODAMINE-conjugated secondary anti-rat IgG antibody. Nuclei were counter-stained with DAPI.

Figure 5 depicts effect of MGC47816-siRNA on the expression of *MGC47816* (a) and effect of MGC47816-siRNA on the viability of Alexander and SNU449 cells (b).

Figure 6 (a) depicts relative expression ratios (cancer/non-cancer) of C2298 in primary 20 HCCs examined by cDNA microarray. Its up-regulated expression (Cy3:Cy5 intensity ratio, >2.0) was observed in 11 of the 12 HCCs that passed through the cutoff filter (both Cy3 and Cy5 signals greater than 25,000). Figure 6 (b) depicts expression of C2298 analysed by semi-quantitative RT-PCR using in eight additional HCCs (T) and their corresponding non-cancerous liver tissues (N). Expression of GAPDH served as an internal control.

Figure 7 depicts the result of multi-tissue northern blot analysis of HES6. The transcript of HES6 is approximately 1.4-kb by size.

Figure 8 depicts the genomic structure of HES6 and the predicted structure of HES6 protein. Exons are indicated by open boxes with nucleotide numbers of HES6 cDNA in the upper panel.

Figure 9 depicts subcellular localization of tagged HES6 protein. (a) Immunoblotting of HA-tagged HES6 protein. (b) Immunohistochemical staining of the tagged protein in COS7 cells. HA-tagged HES6 protein was stained with rat anti-HA monoclonal antibody and visualized by rhodamine-conjugated secondary anti-rat IgG antibody. Nuclei were counter-stained with DAPI.

Figure 10 depicts effect of HES6-siRNA on the expression of HES6 (a) and effect of HES6-siRNA on the viability of Alexander and HepG2 cells (b).

DETAILED DESCRIPTION

The present invention is based in part on the discovery of elevated expression of MGC47816 and HES6 in cells from liver of patients with HCC. These elevated genes expression were identified by using a comprehensive cDNA microarray system.

Using a cDNA microarray containing 23,040 genes, comprehensive gene-expression profiles of 20 patients were constructed previously. *MGC47816* and *HES6* is expressed at high level in HCC patients. In the process candidate molecular marker was selected with the potential of detecting cancer-related proteins in serum or sputum of patients, and some potential targets for development of signal-suppressing strategies in human hepatocellular carcinoma were discovered.

MGC47816 and *HES6* identified herein are used for diagnostic purposes as marker of HCC and as gene target, the expression of which is altered to treat or alleviate a symptom of HCC.

Unless indicated otherwise, "HCC " is meant to refer to any of the sequences disclosed herein.

By measuring expression of MGC47816 or HES6 in a sample of cells, HCC is diagnosed. Similarly, by measuring the expression of MGC47816 or HES6 in response to various agents, and agents for treating HCC can be identified.

The invention involves determining (*e.g.*, measuring) the expression of MGC47816 or HES6. Using sequence information provided by the GeneBank™ database entries for MGC47816 or HES6 sequence, *MGC47816* or *HES6* is detected and measured using techniques well known to one of ordinary skill in the art. For example, sequence within the sequence

database entries corresponding to *MGC47816* or *HES6*, is used to construct probes for detecting MGC47816 or HES6 RNA sequence in, *e.g.*, northern blot hybridization analysis. As another example, the sequences can be used to construct primers for specifically amplifying MGC47816 or HES6 in, *e.g.*, amplification-based detection methods such as reverse-transcription based polymerase chain reaction.

Expression level of MGC47816 or HES6 in the test cell population, *e.g.*, a patient derived tissues sample is then compared to expression level of the MGC47816 or HES6 in a reference population. The reference cell population includes one or more cells for which the compared parameter is known, *i.e.*, HCC cells or non-HCC cells.

Whether or not a pattern of gene expression in the test cell population compared to the reference cell population indicates HCC or a predisposition thereto depends upon the composition of the reference cell population. For example, if the reference cell population is composed of non-HCC cells, a similar gene expression pattern in the test cell population and reference cell population indicates the test cell population is non-HCC. Conversely, if the reference cell population is made up of HCC cells, a similar gene expression profile between the test cell population and the reference cell population indicates that the test cell population includes HCC cells.

A level of expression of a HCC marker gene in a test cell population is considered altered in levels of expression if its expression level varies from the reference cell population by more than 1.0, 1.5, 2.0, 5.0, 10.0 or more fold from the expression level of the corresponding *MGC47816* or *HES6* in the reference cell population.

Differential gene expression between a test cell population and a reference cell population is normalized to a control nucleic acid, *e.g.*, a housekeeping gene. For example, a control nucleic acid is one which is known not to differ depending on the endometriotic or non-endometriotic state of the cell. Expression levels of the control nucleic acid in the test and reference nucleic acid can be used to normalize signal levels in the compared populations. Control genes include ß-actin, glyceraldehyde 3- phosphate dehydrogenase or ribosomal protein P1.

The test cell population is compared to multiple reference cell populations. Each of the multiple reference populations may differ in the known parameter. Thus, a test cell population may be compared to a second reference cell population known to contain, *e.g.*, HCC cells, as well as a second reference population known to contain, *e.g.*, non-HCC cells (normal cells). The test cell is included in a tissue type or cell sample from a subject known to contain, or to be

suspected of containing, HCC cells.

The test cell is obtained from a bodily tissue or a bodily fluid, *e.g.*, biological fluid (such as blood or urine). For example, the test cell is purified from a tissue. Preferably, the test cell population comprises an epithelial cell. The epithelial cell is from tissue known to be or suspected to be a HCC.

Cells in the reference cell population are derived from a tissue type as similar to test cell. Optionally, the reference cell population is a cell line, *e.g.*, a HCC cell line (positive control) or a normal non-HCC cell line (negative control). Alternatively, the control cell population is derived from a database of molecular information derived from cells for which the assayed parameter or condition is known.

The subject is preferably a mammal. The mammal can be, *e.g.*, a human, non-human primate, mouse, rat, dog, cat, horse, or cow.

Expression of MGC47816 or HES6 disclosed herein is determined at the protein or nucleic acid level using methods known in the art. For example, Northern hybridization analysis using probes which specifically recognize the sequence can be used to determine gene expression. Alternatively, expression is measured using reverse-transcription-based PCR assays, *e.g.*, using primers specific for *MGC47816* or *HES6*. Expression is also determined at the protein level, *i.e.*, by measuring the levels of polypeptide encoded by the gene product described herein, or biological activity thereof. Such methods are well known in the art and include, *e.g.*, immunoassays based on antibodies to protein encoded by *MGC47816* or *HES6*. The biological activity of the protein encoded by the gene is also well known.

Diagnosing HCC

HCC is diagnosed by measuring the level of expression of MGC47816 or HES6 from a test population of cells, (*i.e.*, a patient derived biological sample). Preferably, the test cell population contains an epithelial cell, *e.g.*, a cell obtained from liver tissue. Gene expression is also measured from blood or other bodily fluids such as urine. Other biological samples can be used for measuring the protein level. For example, the protein level in the blood, or serum derived from subject to be diagnosed can be measured by immunoassay or biological assay.

Expression of MGC47816 or HES6 is determined in the test cell or biological sample and compared to the expression of the normal control level. A normal control level is an expression profile of MGC47816 or HES6 typically found in a population known not to be suffering from HCC. An increase of the level of expression in the patient derived tissue sample of MGC47816

or HES6 indicates that the subject is suffering from or is at risk of developing HCC.

When *MGC47816* or *HES6* is altered in the test population compared to the normal control level indicates that the subject suffers from or is at risk of developing HCC.

Identifying Agents that inhibit MGC47816 or *HES6 expression or activity*

An agent that inhibits the expression or activity of MGC47816 or HES6 is identified by contacting a test cell population expressing MGC47816 or HES6 with a test agent and determining the expression level or activity of MGC47816 or HES6. A decrease of expression or activity in the presence of the agent compared to the normal control level (or compared to the level in the absence of the test agent) indicates the agent is an inhibitor of MGC47816 or HES6 and useful to inhibit HCC.

The test cell population is any cell expressing MGC47816 or HES6. For example, the test cell population contains an epithelial cell, such as a cell is or derived from liver. For example, the test cell is an immortalized cell line derived from hepatocellular carcinoma. Alternatively, the test cell is a cell, which has been transfected with *MGC47816* or *HES6* or which has been transfected with a regulatory sequence (*e.g.* promoter sequence) from MGC47816 or HES6 operably linked to a reporter gene.

Assessing efficacy of treatment of HCC *in a subject*

The differentially expressed MGC47816 or HES6 identified herein also allow for the course of treatment of HCC to be monitored. In this method, a test cell population is provided from a subject undergoing treatment for HCC. If desired, test cell populations are obtained from the subject at various time points before, during, or after treatment. Expression of MGC47816 or HES6, in the cell population is then determined and compared to a reference cell population which includes cells whose HCC state is known. The reference cells have not been exposed to the treatment.

If the reference cell population contains no HCC cells, a similarity in expression between MGC47816 or HES6 in the test cell population and the reference cell population indicates that the treatment is efficacious. However, a difference in expression between MGC47816 or HES6 in the test population and a normal control reference cell population indicates the less favorable clinical outcome or prognosis.

By "efficacious" is meant that the treatment leads to a reduction in expression of a pathologically up-regulated gene, or a decrease in size, prevalence, or metastatic potential of

hepatocellular tumors in a subject. When treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents HCC from forming or retards, prevents, or alleviates a symptom of clinical HCC. Assessment of hepatocellular tumors are made using standard clinical protocols.

Efficaciousness is determined in association with any known method for diagnosing or treating HCC. HCC is diagnosed for example, by identifying symptomatic anomalies.

Selecting a therapeutic agent for treating HCC *that is appropriate for a particular individual*

Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. An agent that is metabolized in a subject to act as an anti-HCC agent can manifest itself by inducing a change in gene expression pattern in the subject's cells from that characteristic of an HCC state to a gene expression pattern characteristic of a non-HCC state. Accordingly, the differentially expressed MGC47816 or HES6 disclosed herein allow for a putative therapeutic or prophylactic inhibitor of HCC to be tested in a test cell population from a selected subject in order to determine if the agent is a suitable inhibitor of HCC in the subject.

To identify an inhibitor of HCC, that is appropriate for a specific subject, a test cell population from the subject is exposed to a therapeutic agent, and the expression of MGC47816 or HES6 is determined.

The test cell population contains a HCC cell expressing MGC47816 or HES6. Preferably, the test cell is an epithelial cell. For example a test cell population is incubated in the presence of a candidate agent and the pattern of gene expression of the test sample is measured and compared to one or more reference profiles, *e.g.*, a HCC reference expression profile or a non-HCC reference expression profile.

A decrease in expression of MGC47816 or HES6 in a test cell population relative to a reference cell population containing HCC is indicative that the agent is therapeutic.

The test agent can be any compound or composition. For example, the test agents are immunomodulatory agents.

Screening assays for identifying therapeutic agents

MGC47816 or *HES6* disclosed herein can also be used to identify candidate therapeutic agents for treating a HCC. The method is based on screening a candidate therapeutic agent to determine if it converts an expression profile of MGC47816 or HES6 characteristic of a HCC

state to a pattern indicative of a non-HCC state.

In the method, a cell is exposed to a test agent or a combination of test agents (sequentially or consequentially) and the expression of MGC47816 or HES6 in the cell is measured. The expression level of MGC47816 or HES6 in the test population is compared to expression level of MGC47816 or HES6 in a reference cell population that is not exposed to the test agent.

An agent effective in suppressing expression of over-expressed genes is deemed to lead to a clinical benefit. Such compounds are further tested for the ability to prevent HCC growth.

In a further embodiment, the present invention provides methods for screening candidate agents which are potential targets in the treatment of HCC. As discussed in detail above, by controlling the expression levels or activities of marker gene, one can control the onset and progression of HCC. Thus, candidate agents, which are potential targets in the treatment of HCC, can be identified through screenings that use the expression levels and activities of marker gene as indices. In the context of the present invention, such screening may comprise, for example, the following steps:

a) contacting a test compound with a polypeptide encoded by a nucleic acid of MGC47816 or HES6;
b) detecting the binding activity between the polypeptide and the test compound; and
c) selecting a compound that binds to the polypeptide

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell expressing MGC47816 or HES6, and
b) selecting a compound that reduces the expression level of MGC47816 or HES6.

Cells expressing marker gene include, for example, cell lines established from HCC; such cells can be used for the above screening of the present invention.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a test compound with a polypeptide encoded by a nucleic acid of MGC47816 or HES6;
b) detecting the biological activity of the polypeptide of step (a); and
c) selecting a compound that suppresses the biological activity of the polypeptide encoded by a nucleic acid of MGC47816 or HES6 in comparison with the biological activity detected in the absence of the test compound.

A protein required for the screening can be obtained as a recombinant protein using the nucleotide sequence of the marker gene. Based on the information of the marker gene, one skilled in the art can select any biological activity of the protein as an index for screening and a measurement method based on the selected biological activity. Preferably, cell proliferative activity of MGC47816 or HES6 may be selected as the biological activity. The cell proliferative activity is detected by proliferation of cell line such as NIH3T3, COS7.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of MGC47816 or HES6 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene, as compared to a control.

Suitzable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared by using the transcriptional regulatory region of a marker gene. When the transcriptional regulatory region of a marker gene has been known to those skilled in the art, a reporter construct can be prepared by using the previous sequence information. When the transcriptional regulatory region of a marker gene remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the marker gene.

The compound isolated by the screening is a candidate for drugs that inhibit the activity of the protein encoded by marker gene and can be applied to the treatment or prevention of HCC.

Moreover, compound in which a part of the structure of the compound inhibiting the activity of protein encoded by marker gene is converted by addition, deletion and/or replacement are also included in the compounds obtainable by the screening method of the present invention.

When administrating the compound isolated by the method of the invention as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees, the isolated compound can be directly administered or can be formulated into a dosage form using known pharmaceutical preparation methods. For example, according to the need, the drugs can be taken orally, as sugar-coated tablets, capsules, elixirs and microcapsules, or non-orally, in the form of injections

of sterile solutions or suspensions with water or any other pharmaceutically acceptable liquid. For example, the compounds can be mixed with pharmaceutically acceptable carriers or media, specifically, sterilized water, physiological saline, plant-oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binders, and such, in a unit dose form required for generally accepted drug implementation. The amount of active ingredients in these preparations makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be mixed to tablets and capsules are, binders such as gelatin, corn starch, tragacanth gum and arabic gum; excipients such as crystalline cellulose; swelling agents such as corn starch, gelatin and alginic acid; lubricants such as magnesium stearate; sweeteners such as sucrose, lactose or saccharin; and flavoring agents such as peppermint, Gaultheria adenothrix oil and cherry. When the unit-dose form is a capsule, a liquid carrier, such as an oil, can also be further included in the above ingredients. Sterile composites for injections can be formulated following normal drug implementations using vehicles such as distilled water used for injections.

Physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injections. These can be used in conjunction with suitable solubilizers, such as alcohol, specifically ethanol, polyalcohols such as propylene glycol and polyethylene glycol, non-ionic surfactants, such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or Soy-bean oil can be used as a oleaginous liquid and may be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizer and may be formulated with a buffer, such as phosphate buffer and sodium acetate buffer; a pain-killer, such as procaine hydrochloride; a stabilizer, such as benzyl alcohol and phenol; and an anti-oxidant. The prepared injection may be filled into a suitable ampule.

Methods well known to one skilled in the art may be used to administer the pharmaceutical composition of the present inevntion to patients, for example as intraarterial, intravenous, or percutaneous injections and also as intranasal, transbronchial, intramuscular or oral administrations. The dosage and method of administration vary according to the body-weight and age of a patient and the administration method; however, one skilled in the art can routinely select a suitable metod of administration. If said compound is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to a patient to perform the therapy. The dosage and method of administration vary according to the body-weight, age, and symptoms of the patient but one skilled in the art can suitably select them.

Hepatocellular Carcinoma

For example, although the dose of a compound that binds to the protein of the present invention and regulates its activity depends on the symptoms, the dose is about 0.1 mg to about 100 mg per day, preferably about 1.0 mg to about 50 mg per day and more preferably about 1.0 mg to about 20 mg per day, when administered orally to a normal adult (weight 60 kg).

When administering parenterally, in the form of an injection to a normal adult (weight 60 kg), although there are some differences according to the patient, target organ, symptoms and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, preferably about 0.1 to about 20 mg per day and more preferably about 0.1 to about 10 mg per day. Also, in the case of other animals too, it is possible to administer an amount converted to 60 kgs of body-weight.

Assessing the prognosis of a subject with HCC

Also provided is a method of assessing the prognosis of a subject with HCC by comparing the expression of MGC47816 or HES6 in a test cell population to the expression of the gene in a reference cell population derived from patients over a spectrum of disease stages. By comparing gene expression of MGC47816 or HES6 in the test cell population and the reference cell population(s), or by comparing the pattern of gene expression over time in test cell populations derived from the subject, the prognosis of the subject can be assessed.

An increase of expression of MGC47816 or HES6 compared to a normal control indicates less favorable prognosis. A similarity in expression of MGC47816 or HES6 indicates a more favorable prognosis for the subject.

Kits

The invention also includes a HCC-detection reagent, *e.g.*, a nucleic acid that specifically binds to or identifies MGC47816 or HES6 nucleic acids such as oligonucleotide sequences, which are complementary to a portion of a MGC47816 or HES6 nucleic acid or antibodies which bind to proteins encoded by a MGC47816 or HES6 nucleic acid. The reagents are packaged together in the form of a kit. The reagents are packaged in separate containers, *e.g.*, a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix), a control reagent (positive and/or negative), and/or a detectable label. Instructions (*e.g.*, written, tape, CD-ROM, etc.) for carrying out the assay are included in the kit. The assay format of the kit is a Northern hybridization or a sandwich ELISA known in the art.

For example, HCC detection reagent is immobilized on a solid matrix such as a porous

strip to form at least one HCC detection site. The measurement or detection region of the porous strip may include a plurality of sites containing a nucleic acid. A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites are located on a separate strip from the test strip. Optionally, the different detection sites may contain different amounts of immobilized nucleic acids, *i.e.*, a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of HCC present in the sample. The detection sites may be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a teststrip.

Methods of inhibiting HCC

The invention provides a method for treating or alleviating a symptom of HCC in a subject by decreasing expression or activity of MGC47816 or HES6. Therapeutic compounds are administered prophylactically or therapeutically to subject suffering from (or susceptible to) developing HCC. Administration can be systemic or local. Such subjects are identified using standard clinical methods or by detecting an aberrant level of expression or activity of MGC47816 or HES6. Therapeutic agents include inhibitors of cell proliferation.

The method includes decreasing the expression, or function, or both, of gene products of MGC47816 or HES6. Expression is inhibited in any of several ways known in the art. For example, expression is inhibited by administering to the subject a nucleic acid that inhibits, or antagonizes, the expression of the over-expressed gene, *e.g.*, an antisense oligonucleotide or small interfering RNA which disrupts expression of the over-expressed gene.

As noted above, antisense nucleic acids corresponding to the nucleotide sequence of MGC47816 or HES6 can be used to reduce the expression level of the MGC47816 or HES6. Antisense nucleic acids corresponding to the nucleotide sequence of MGC47816 or HES6 that are up-regulated in HCC are useful for the treatment of HCC. Specifically, the antisense nucleic acids of the present invention may act by binding to the nucleotide sequence of MGC47816 or HES6 or mRNA corresponding thereto, thereby inhibiting the transcription or translation of the gene, promoting the degradation of the mRNA, and/or inhibiting the expression of protein encoded by a nucleic acid of MGC47816 or HES6, finally inhibiting the function of the proteins. The term "antisense nucleic acids" as used herein encompasses both nucleotides that are entirely complementary to the target sequence and those having a mismatch of nucleotide, so long as the antisense nucleic acids can specifically hybridize to the target

sequences. For example, the antisense nucleic acids of the present invention include polynucleotides that have a homology of at least 70% or higher, preferably at 80% or higher, more preferably 90% or higher, even more preferably 95% or higher over a span of at least 15 continuous nucleotides. Algorithms known in the art can be used to determine the homology.

The antisense nucleic acid derivatives of the present invention act on cells producing the protein encoded by marker gene by binding to the DNA or mRNA encoding the protein, inhibiting their transcription or translation, promoting the degradation of the mRNA, and inhibiting the expression of the protein, thereby resulting in the inhibition of the protein function.

An antisense nucleic acid derivative of the present invention can be made into an external preparation, such as a liniment or a poultice, by mixing with a suitable base material which is inactive against the derivative.

Also, as needed, the derivatives can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, stabilizers, preservatives, pain-killers, and such. These can be prepared by following known methods.

The antisense nucleic acids derivative is given to the patient by directly applying onto the ailing site or by injecting into a blood vessel so that it will reach the site of ailment. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples are, liposomes, poly-L-lysine, lipids, cholesterol, lipofectin or derivatives of these.

The dosage of the antisense nucleic acid derivative of the present invention can be adjusted suitably according to the patient's condition and used in desired amounts. For example, a dose range of 0.1 to 100 mg/kg, preferably 0.1 to 50 mg/kg can be administered.

The antisense nucleic acids of the invention inhibit the expression of the protein of the invention and is thereby useful for suppressing the biological activity of a protein of the invention. Also, expression-inhibitors, comprising the antisense nucleic acids of the invention, are useful since they can inhibit the biological activity of a protein of the invention.

The antisense nucleic acids of present invention include modified oligonucleotides. For example, thioated nucleotides may be used to confer nuclease resistance to an oligonucleotide.

Also, a siRNA against marker gene can be used to reduce the expression level of the marker gene. By the term "siRNA" is meant a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is transcribed. In the context of the present invention, the siRNA comprises a sense nucleic acid sequence and an anti-sense

nucleic acid sequence against an upregulated marker gene, such as *MGC47816* or *HES6*. The siRNA is constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, *e.g.*, a hairpin.

The method is used to alter the expression in a cell of an upregulated, *e.g.*, as a result of malignant transformation of the cells. Binding of the siRNA to a transcript corresponding to MGC47816 or HES6 in the target cell results in a reduction in the protein production by the cell. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring the transcript. Preferably, the oligonucleotide is 19-25 nucleotides in length. Most preferably, the oligonucleotide is less than 75, 50 , 25 nucleotides in length. Examples of MGC47816 siRNA oligonucleotide which inhibited the expression in Alexander and SNU449 cells include the target sequence containing SEQ ID NO: 21 and 22. Examples of HES6 siRNA oligonucleotide which inhibited the expression in Alexander and HepG2 cells include the target sequence containing SEQ ID NO: 31 and 32.

The nucleotide sequence of the siRNAs were designed using a siRNA design computer program available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). The computer program selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the object transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl, et al. recommend against designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with the binding of the siRNA endonuclease complex.
2. Compare the potential target sites to the human genome database and eliminate from consideration any target sequences with significant homology to other coding sequences. The homology search can be performed using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/
3. Select qualifying target sequences for synthesis. At Ambion, preferably several target sequences can be selected along the length of the gene for evaluation.

The antisense oligonucleotide or siRNA of the invention inhibit the expression of the polypeptide of the invention and is thereby useful for suppressing the biological activity of the

polypeptide of the invention. Also, expression-inhibitors, comprising the antisense oligonucleotide or siRNA of the invention, are useful in the point that they can inhibit the biological activity of the polypeptide of the invention. Therefore, a composition comprising the antisense oligonucleotide or siRNA of the present invention are useful in treating a HCC.

Alternatively, function of gene product of the over-expressed gene is inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. For example, the compound is an antibody which binds to the over-expressed gene product.

The present invention refers to the use of antibodies, particularly antibodies against a protein encoded by an up-regulated marker gene, or a fragment of the antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure, that interacts (i.e., binds) only with the antigen that was used for synthesizing the antibody (i.e., the up-regulated marker gene product) or with an antigen closely related to it. Furthermore, an antibody may be a fragment of an antibody or a modified antibody, so long as it binds to the protein encoded by the marker gene. For instance, the antibody fragment may be Fab, $F(ab')_2$, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston J. S. et al. Proc. Natl. Acad. Sci. U.S.A. 85:5879-5883 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment may be constructed, inserted into an expression vector, and expressed in an appropriate host cell (see, for example, Co M. S. et al. J. Immunol. 152:2968-2976 (1994); Better M. and Horwitz A. H. Methods Enzymol. 178:476-496 (1989); Pluckthun A. and Skerra A. Methods Enzymol. 178:497-515 (1989); Lamoyi E. Methods Enzymol. 121:652-663 (1986); Rousseaux J. et al. Methods Enzymol. 121:663-669 (1986); Bird R. E. and Walker B. W. Trends Biotechnol. 9:132-137 (1991)).

An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). The present invention provides such modified antibodies. The modified antibody can be obtained by chemically modifying an antibody. These modification methods are conventional in the field.

Alternatively, an antibody may be obtained as a chimeric antibody, between a variable region derived from a nonhuman antibody and a constant region derived from a human antibody, or as a humanized antibody, comprising the complementarity determining region (CDR) derived from a nonhuman antibody, the frame work region (FR) derived from a human antibody, and the constant region. Such antibodies can be prepared by using known technologies.

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-cancer drugs such as trastuzumab (Herceptin) for the treatment of advanced breast cancer, imatinib methylate (Gleevec) for chronic myeloid leukemia, gefitinib (Iressa) for non-small cell lung cancer (NSCLC), and rituximab (anti-CD20 mAb) for B-cell lymphoma and mantle cell lymphoma (Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res. 2001 Oct;7(10):2958-70. Review.; Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783-92.; Rehwald U, Schulz H, Reiser M, Sieber M, Staak JO, Morschhauser F, Driessen C, Rudiger T, Muller-Hermelink K, Diehl V, Engert A. Treatment of relapsed CD20+ Hodgkin lymphoma with the monoclonal antibody rituximab is effective and well tolerated: results of a phase 2 trial of the German Hodgkin Lymphoma Study Group. Blood. 2003 Jan 15;101(2):420-424.; Fang G, Kim CN, Perkins CL, Ramadevi N, Winton E, Wittmann S and Bhalla KN. (2000). Blood, 96, 2246-2253.). These drugs are clinically effective and better tolerated than traditional anti-cancer agents because they target only transformed cells. Hence, such drugs not only improve survival and quality of life for cancer patients, but also validate the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination with it (Gianni L. (2002). Oncology, 63 Suppl 1, 47-56.; Klejman A, Rushen L, Morrione A, Slupianek A and Skorski T. (2002). Oncogene, 21, 5868-5876.). Therefore, future cancer treatments will probably involve combining conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness.

These modulatory methods are performed *ex vivo* or *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). The method involves administering a protein or combination of proteins or a nucleic acid molecule or combination of nucleic acid, molecules as therapy to counteract aberrant expression or activity of the differentially expressed genes.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) levels or biological activity of the genes may be treated with therapeutics that antagonize (*i.e.*, reduce or inhibit) activity of the over-expressed gene or genes. Therapeutics that antagonize activity are administered therapeutically or

prophylactically.

Therapeutics that may be utilized include, *e.g.*, (*i*) antibodies to the MGC47816 or HES6; (*ii*) antisense nucleic acids or nucleic acids that are "dysfunctional" (*i.e.*, due to a heterologous insertion within the coding sequence of MGC47816 or HES6 sequence); (*iii*) small interfering RNA (siRNA); or (*iv*) modulators (*i.e.*, inhibitors or antagonists that alter the interaction between an MGC47816 or HES6 polypeptide and its binding partner. The dysfunctional antisense molecule is utilized to "knockout" endogenous function of a polypeptide by homologous recombination (see, *e.g.*, Capecchi, *Science* 244: 1288-1292 1989).

Increased level can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it *in vitro* for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a gene whose expression is altered). Methods that are well-known within the art include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, *in situ* hybridization, etc.).

Prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

Therapeutic methods include contacting a cell with an agent that modulates one or more of the activities of the gene product of the differentially expressed gene. An agent that modulates protein activity includes a nucleic acid or a protein, a naturally-occurring cognate ligand of these proteins, a peptide, a peptidomimetic, or other small molecule.

The present invention also relates to a method of treating or preventing HCC in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid of MGC47816 or HES6 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide or the fragment thereof. An administration of the polypeptide induce an anti-tumor immunity in a subject. To inducing anti-tumor immunity, a polypeptide encoded by a nucleic acid of MGC47816 or HES6 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide is administered. The polypeptide or the immunologically active fragments thereof are useful as vaccines against HCC. In some cases the proteins or fragments thereof may be administered in a form bound to the T cell recepor (TCR) or presented by an antigen presenting cell (APC), such as macrophage, dendritic cell (DC), or B-cells. Due to the strong antigen presenting ability of DC, the use of

DC is most preferable among the APCs.

In the present invention, vaccine against HCC refers to a substance that has the function to induce anti-tumor immunity upon inoculation into animals. According to the present invention, polypeptides encoded by a nucleic acid of MGC47816 or HES6 or fragments thereof were suggested to be HLA-A24 or HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against HCC cells expressing MGC47816 or HES6. Thus, the present invention also encompasses method of inducing anti-tumor immunity using the polypeptides. In general, anti-tumor immunity includes immune responses such as follows:

- induction of cytotoxic lymphocytes against tumors,
- induction of antibodies that recognize tumors, and
- induction of anti-tumor cytokine production.

Therefore, when a certain protein induces any one of these immune responses upon inoculation into an animal, the protein is decided to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing *in vivo* or *in vitro* the response of the immune system in the host against the protein.

For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. A foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by APC in antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to T cell by APC, and detecting the induction of CTL. Furthermore, APC has the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells and CD8+ T cells are also important in anti-tumor immunity, the anti-tumor immunity inducing action of the peptide can be evaluated using the activation effect of these cells as indicators.

A method for evaluating the inducing action of CTL using dendritic cells (DCs) as APC is well known in the art. DC is a representative APC having the strongest CTL inducing action among APCs. In this method, the test polypeptide is initially contacted with DC, and then this DC is contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTL against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the method of evaluating the degree

of tumor cell damage using ^{3}H-thymidine uptake activity or LDH (lactose dehydrogenase)-release as the indicator is also well known.

Apart from DC, peripheral blood mononuclear cells (PBMCs) may also be used as the APC. The induction of CTL is reported that the it can be enhanced by culturing PBMC in the presence of GM-CSF and IL-4. Similarly, CTL has been shown to be induced by culturing PBMC in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

The test polypeptides confirmed to possess CTL inducing activity by these methods are polypeptides having DC activation effect and subsequent CTL inducing activity. Therefore, polypeptides that induce CTL against tumor cells are useful as vaccines against tumors. Furthermore, APC that acquired the ability to induce CTL against tumors by contacting with the polypeptides are useful as vaccines against tumors. Furthermore, CTL that acquired cytotoxicity due to presentation of the polypeptide antigens by APC can be also used as vaccines against tumors. Such therapeutic methods for tumors using anti-tumor immunity due to APC and CTL are referred to as cellular immunotherapy.

Generally, when using a polypeptide for cellular immunotherapy, efficiency of the CTL-induction is known to increase by combining a plurality of polypeptides having different structures and contacting them with DC. Therefore, when stimulating DC with protein fragments, it is advantageous to use a mixture of multiple types of fragments.

Alternatively, the induction of anti-tumor immunity by a polypeptide can be confirmed by observing the induction of antibody production against tumors. For example, when antibodies against a polypeptide are induced in a laboratory animal immunized with the polypeptide, and when growth of tumor cells is suppressed by those antibodies, the polypeptide can be determined to have an ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccine of this invention, and the induction of anti-tumor immunity enables treatment and prevention of HCC. Therapy against cancer or prevention of the onset of cancer includes any of the steps, such as inhibition of the growth of cancerous cells, involution of cancer, and suppression of occurrence of cancer. Decrease in mortality of individuals having cancer, decrease of tumor markers in the blood, alleviation of detectable symptoms accompanying cancer, and such are also included in the therapy or prevention of cancer. Such therapeutic and preventive effects are preferably statistically significant. For example, in observation, at a significance level of 5% or less, wherein the therapeutic or preventive effect of a vaccine against cell proliferative diseases is compared to a control without vaccine administration. For example, Student's t-test, the Mann-

Whitney U-test, or ANOVA may be used for statistical analysis.

The above-mentioned protein having immunological activity or a vector encoding the protein may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the protein when administered together (or successively) with the protein having immunological activity. Examples of adjuvants include cholera toxin, salmonella toxin, alum, and such, but are not limited thereto. Furthermore, the vaccine of this invention may be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers are sterilized water, physiological saline, phosphate buffer, culture fluid, and such. Furthermore, the vaccine may contain as necessary, stabilizers, suspensions, preservatives, surfactants, and such. The vaccine is administered systemically or locally. Vaccine administration may be performed by single administration, or boosted by multiple administrations.

When using APC or CTL as the vaccine of this invention, tumors can be treated or prevented, for example, by the *ex vivo* method. More specifically, PBMCs of the subject receiving treatment or prevention are collected, the cells are contacted with the polypeptide *ex vivo*, and following the induction of APC or CTL, the cells may be administered to the subject. APC can be also induced by introducing a vector encoding the polypeptide into PBMCs *ex vivo*. APC or CTL induced *in vitro* can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APC and CTL isolated in this manner may be used for cellular immunotherapy not only against individuals from whom the cells are derived, but also against similar types of tumors from other individuals.

Furthermore, a pharmaceutical composition for treating or preventing a cell proliferative disease, such as cancer, comprising a pharmaceutically effective amount of the polypeptide of the present invention is provided. The pharmaceutical composition may be used for raising anti tumor immunity.

Pharmaceutical compositions for inhibiting HCC

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for administration by inhalation or insufflation. Preferably, administration is intravenous. The formulations are optionally packaged in discrete dosage units.

Pharmaceutical formulations suitable for oral administration include capsules, cachets or tablets, each containing a predetermined amount of the active ingredient. Formulations also include powders, granules or solutions, suspensions or emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrant or wetting agents. A tablet may be made by compression or molding, optionally with one or more formulational ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be coated according to methods well known in the art. Oral fluid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives. The tablets may optionally be formulated so as to provide slow or controlled release of the active ingredient therein. A package of tablets may contain one tablet to be taken on each of the month.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations may be presented for continuous infusion. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges, which contain the active ingredient in a flavored base such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a base such as gelatin and glycerin or sucrose and acacia. For intra-nasal

administration the compounds of the invention may be used as a liquid spray or dispersible powder or in the form of drops. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents.

For administration by inhalation the compounds are conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichiorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds may take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form, in for example, capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflators.

Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

When desired, the above described formulations, adapted to give sustained release of the active ingredient, may be employed. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

Preferred unit dosage formulations are those containing an effective dose, as recited below, or an appropriate fraction thereof, of the active ingredient.

For each of the aforementioned conditions, the compositions, *e.g.*, polypeptides and organic compounds are administered orally or via injection at a dose of from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, preferably about 5 mg to about 10 g/day, and most preferably about 100 mg to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units may conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

Hepatocellular Carcinoma

The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration may vary depending upon the condition and its severity.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLE 1 MATERIALS AND METHODS

Patients and tissue specimens

All hepatocellular carcinoma tissues and the corresponding non-cancerous tissues were obtained with informed consent from surgical specimens of patients who underwent surgery.

Genome-wide cDNA microarray

In this study, we used our in-house genome-wide cDNA microarray with 23040 genes. Total RNA extracted from the microdissected tissue was treated with DNase I, amplified with Ampliscribe T7 Transcription Kit (Epicentre Technologies), and subsequently labeled during reverse transcription with Cy-dye (Amersham); RNA from non-cancerous tissue with Cy5 and RNA from tumor with Cy3. Hybridization, washing, and detection were carried out as described previously (4), and fluorescence intensity of Cy5 and Cy3 for each target spot was generated by ArrayVision software (Amersham Pharmacia). After subtraction of background signal, the duplicate values were averaged for each spot. Then, all fluorescence intensities on a slide were normalized to adjust the mean Cy5 and Cy3 intensities of 52 housekeeping genes for each slide. Genes were excluded from further investigation when the intensities of both Cy3 and Cy5 were below 25,000 fluorescence units, and of the remainder, we selected for further evaluation those with Cy3/Cy5 signal ratios > 2.0.

Cell lines

Human hepatoma cell lines Alexander and HepG2 and monkey fibroblast cell line COS7 were obtained from the American Type Culture Collection (ATCC). Another human hepatoma cell line Huh7 was obtained from Japanese Collection of Research Bioresources (JCRB), while SNU423, SNU449 and SNU475 were obtained from the Korea cell-line bank. All cell lines were grown in monolayers in appropriate media: Dulbecco's modified Eagle's medium for Alexander, Huh7, HepG2 and COS7; RPMI1640 for SNU423, SNU449 and SNU475 supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution (Sigma).

All cells were maintained at 37°C in humid air with 5% CO_2, (Alexander, Huh7, HepG2, SNU423, SNU449, SNU475, and COS7).

RNA preparation and RT-PCR

Total RNA was extracted with a Qiagen RNeasy kit (Qiagen) or Trizol reagent (Life Technologies, Inc.) according to the manufacturers' protocols. Ten-microgram aliquots of total RNA were reversely transcribed for single-stranded cDNAs using poly $dT_{12\text{-}18}$ primer (Amersham Pharmacia Biotech) with Superscript II reverse transcriptase (Life Technologies). Each single-stranded cDNA preparation was diluted for subsequent PCR amplification by standard RT-PCR experiments carried out in 12 µl volumes of PCR buffer (TAKARA). Amplification proceeded for 4 min at 94°C for denaturing, followed by 21 (for *GAPDH*), 35 (for *MGC47816*) cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s, and 35 (for HES6) cycles of 94°C for 30 s, 60°C for 40 s, and 72°C for 60 s, in the GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, CA). Primer sequences were; for *GAPDH:* forward, 5'-ACAACAGCCTCAAGATCATCAG-3' (SEQ ID NO: 3) and reverse, 5'-GGTCCACCACTGACACGTTG-3' (SEQ ID NO: 4); for *MGC47816*: forward, 5'-CAAATAGGCAGACTGGAAAGATG-3' (SEQ ID NO: 5) and reverse: 5'-CTAGGGAAGCAGTAGGATTTGGT-3' (SEQ ID NO: 6); for HES6: forward, 5'-GAGCTCCTGAACCATCTGCTC-3' (SEQ ID NO: 23) and reverse: 5'-CAAGATGTACAGAGCATCACAGC-3' (SEQ ID NO: 24);

Northern-blot analysis

Human multiple-tissue blots (Clontech, Palo Alto, CA) were hybridized with a ^{32}P-labeled PCR product of *MGC47816* and *HES6*. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at −80°C for 72 h.

Construction of expression vector

The entire coding region of MGC47816 was amplified by RT-PCR using gene specific sets of primers; 5'-ATTGTCGACGCTCGCCCTACTGAGCGAGCG-3' (SEQ ID NO: 7), and 5'-AATCTCGAGAGCAGGAATTCACTTAAGTTTTAACTC-3' (SEQ ID NO: 8).

The entire coding region of HES6 was amplified by RT-PCR using a gene-specific set of primers; 5'-ATTGAATTCGCATGGCGCCACCCGCGGCG-3' (SEQ ID NO: 25), and 5'-

AATGGTACCTCACCAAGGCCTCCAGACACTCC-3' (SEQ ID NO: 26). The PCR product was cloned into an appropriate cloning site of pCMV-HA vector (CLONTECH). The PCR product was cloned into appropriate cloning site of pCMV-HA(Clontech) vector.

Immunoblotting

Cells transfected with pCMV-HA-MGC47816 and pCMV-HA-HES6 were washed twice with PBS and harvested in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 1 mM DTT, and 1X complete Protease Inhibitor Cocktail (Boehringer)). After the cells were homogenized and centrifuged at 10,000x*g* for 30 min, the supernatant was standardized for protein concentration by the Bradford assay (Bio-Rad). Proteins were separated by 10% SDS-PAGE and immunoblotted with rat anti-HA (Roche) antibody. HRP-conjugated goat anti-rat IgG (Santa Cruz) served as the secondary antibody for the ECL Detection System (Amersham).

Immunohistochemical staining

Cells transfected with pCMV-HA-MGC47816 and pCMV-HA-HES6 was fixed with PBS containing 4% paraformaldehyde for 15 min, then rendered permeable with PBS containing 0.1% Triton X-100 for 2.5 min at RT. Subsequently the cells were covered with 2% BSA in PBS for 12 h at 4°C to block non-specific hybridization. Rat anti-HA (ROCHE) antibody at 1:1000 dilution was used for the first antibody, and the reaction was visualized after incubation with RHODAMINE-conjugated anti-rat second antibody (Leinco and ICN). Nuclei were counter-stained with 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under an ECLIPSE E800 microscope.

Construction and effect of plasmids expressing MGC47816-siRNA and HES6-siRNA

To prepare plasmid vector expressing short interfering RNA (siRNA), we amplified the genomic fragment of *H1RNA* gene containing its promoter region by PCR using a set of primers, 5'-TGGTAGCCAAGTGCAGGTTATA-3' (SEQ ID NO: 9) and 5'-CCAAAGGGTTTCTGCAGTTTCA-3' (SEQ ID NO: 10) for *H1RNA* and human placental DNA as a template. The products were purified and cloned into pCR2.0 plasmid vector using a TA cloning kit according to the supplier's protocol (Invitrogen). The *Bam*HI and *Xho*I fragment containing *H1RNA* was into pcDNA3.1(+) between nucleotides 56 and 1257, which was amplified by PCR using 5'-TGCGGATCCAGAGCAGATTGTACTGAGAGT-3' (SEQ ID NO: 11) and 5'-CTCTATCTCGAGTGAGGCGGAAAGAACCA-3' (SEQ ID NO: 12). The

ligated DNA became the template for PCR amplification with primers, 5'-TTTAAGCTTGAAGACCATTTTTGGAAAAAAAAAAAAAAAAAAAAC-3' (SEQ ID NO: 13) and 5'-TTTAAGCTTGAAGACATGGGAAAGAGTGGTCTCA-3' (SEQ ID NO: 14) for *H1RNA*. The product was digested with *Hin*dIII, and subsequently self-ligated to produce psiH1BX3.0 or psiU6BX3.0 vector plasmids. Control plasmid, psiH1BX-EGFP was prepared by cloning double-stranded oligonucleotides of 5'-CACCGAAGCAGCACGACTTCTTCTTCAAGAGAGAAGAAGTCGTGCTGCTTC-3' (SEQ ID NO: 15) and 5'-AAAAGAAGCAGCACGACTTCTTCTCTCTTGAAGAAGAAGTCGTGCTGCTTC-3' (SEQ ID NO: 16) into the *Bbs*I site in the psiH1BX3.0 vector. Plasmids expressing MGC47816-siRNAs and HES6-siRNAs were prepared by cloning of double-stranded oligonucleotides into psiH1BX3.0 vector. The oligonucleotides used for MGC47816-siRNAs were 5'-TCCCCATCACCTCTCTGACATCTTTCAAGAGAAGATGTCAGAGAGGTGATG-3' (SEQ ID NO: 17) and 5'-AAAACATCACCTCTCTGACATCTTCTCTTGAAAGATGTCAGAGAGGTGATG-3' (SEQ ID NO: 18) (psiH1BX-MGC47816-2), and 5'-TCCCGTGTCCGCTGACAGAACAATTCAAGAGATTGTTCTGTCAGCGGACAC-3' (SEQ ID NO: 19) and 5'-AAAAGTGTCCGCTGACAGAACAATCTCTTGAATTGTTCTGTCAGCGGACAC-3' (SEQ ID NO: 20) (psiH1BX-MGC47816-3).

The oligonucleotides used for HES6-siRNAs were 5'-TCCCACTTTTAGGGACCCTGCAGTTCAAGAGACTGCAGGGTCCCTAAAAGT-3' (SEQ ID NO: 27) and 5'-AAAAACTTTTAGGGACCCTGCAGTCTCTTGAACTGCAGGGTCCCTAAAAGT-3' (SEQ ID NO: 28) (psiH1BX-HES6-2), and 5'-TCCCCAAAAGCTTGAACTTGCCATTCAAGAGATGGAAAGTTCAAGCTTTTG-3' (SEQ ID NO: 29) and 5'-AAAACAAAAGCTTGAACTTGCCATCTCTTGAATGGCAAGTTCAAGCTTTTG-3' (SEQ ID NO: 30) (psiH1BX-HES6-3).

Plasmids, psiH1BX-MGC47816-2 and psiH1BX-MGC47816-3, were transfected into Alexander and SNU449 cells, psiH1BXNo2-HES6 and psiH1BX-No3-HES6 were transfected into Alexander and HepG2 cells using FuGENE6 reagent (Roche) or Nucleofector reagent (Alexa) according to the supplier's recommendations. Total RNA was extracted from the cells

48 hours after the transfection. Cells were cultured in the presence of 400-800 μg/ml geneticin (G418) for 14 days and stained with Giemsa's solution (MERCK, Germany) as described elsewhere.

MTT assay

Cells ($1x10^6$) on 10cm-dish were transfected with expression vector or control vector using FuGene6 (Roche) according to the supplier's protocol. Cell viability was evaluated by MTT assay seven days after transfection. Cell-counting kit-8 (DOJINDO) was added to each dish at a concentration of 1/10 volume, and the plates were incubated at 37°C for an additional 2 h; then absorbance was measured at 490 nm, and at 630 nm as reference, with a Microplate Reader 550 (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the Scheffé's F test.

EXAMPLE 2. RESULTS

Identification of D4999 whose expression is frequently up-regulated in human HCC

We compared expression profiles of 20 HCCs with their corresponding non-cancerous liver tissues using cDNA microarray containing 23040 genes. Among commonly up-regulated genes in HCCs, a gene with an in-house accession number of D4999, corresponding to an EST (MGC47816) in Hs.420244 of a UniGene cluster (http://www.ncbi. nlm.nih.gov/UniGene/), was over-expressed in seven of eleven HCCs compared with the corresponding noncancerous liver tissues (Figure1). To clarify the results of the microarray, we carried out semi-quantitative RT-PCR and revealed that expression of D4999 was increased in seven of additional eight HCCs compared with their corresponding non-cancerous liver tissues (Figure 2).

Identification, expression, and structure of *MGC47816*

Homology searches with the sequence of D4999 in public databases using BLAST program in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) identified ESTs including *MGC47816* (GenBank accession number of NM_173642) and a genomic sequence with GenBank accession number of AA971400 assigned to chromosomal band 1q34.1. Comparison of *MGC47816* cDNA and the genomic sequence disclosed that this gene consisted of 5 exons. The peutative full-length

cDNA consisted of 1528 nucleotides, with an open reading frame of 1176 nucleotides (SEQ ID NO: 1) encoding a 391-amino-acid protein (SEQ ID NO: 2). The amino acid sequence of the predicted MGC47816 protein showed 88 % identity to a mouse hypothetical protein B930030J24. A search for protein motifs with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) revealed that the predicted protein contained a carbamoyl-phosphate synthase L chain and an ATP binding domain (codons 71-253) (Figure 3).

Subcellular localization of HA-tagged MGC47816 protein

To investigate the subcellular localization of MGC47816 protein, we transiently transfected a plasmid expressing HA-tagged (pCMV-HA-MGC47816) COS7 cells. Western blot analysis using extracts from the cells and anti-HA antibody revealed a 50-KDa band corresponding to the tagged protein (Figure 4a). Subsequent immunohistochemical staining of the cells with these antibodies indicated that the protein was mainly present in the cytoplasm (Figure 4b).

Effect of plasmids expressing MGC47816-siRNA on growth of HCC cells

To investigate the function of MGC47816 in cancer cells, we constructed plasmids expressing MGC47816-siRNA and examined their effect on *MGC47816* expression. Transfection of Alexander and SNU449 cells with psiH1BX-MGC47816-2 (Si-2), psiH1BX-MGC47816-3 (Si-3), psiH1BX-EGFP (EGFP) or psiH1BX-mock (Mock) revealed that psiH1BX-MGC47816-3 (Si-3) significantly suppressed expression of *MGC47816* in the cells compared to MGC47816-2 (Si-2), psiH1BX-EGFP (EGFP) or psiH1BX-mock (Mock) (Figure 5a). To test whether the suppression of *MGC47816* may result in growth suppression of hepatocellular carcinoma cells, we transfected Alexander and SNU449 cells with psiH1BX-MGC47816-2 (Si-2), psiH1BX-MGC47816-3 (Si-3), psiH1BX-EGFP (EGFP) or psiH1BX-mock (Mock). Viable cells transfected with psiH1BX-MGC47816-3 (Si-3) were markedly reduced compared to those transfected with psiH1BX-MGC47816-2 (Si-2), psiH1BX-EGFP (EGFP) or psiH1BX-mock (Mock) suggesting that decreased expression of *MGC47816* suppressed growth of hepatocellular carcinoma cells (Figure 5b).

Identification of C2298 whose expression was frequently elevated in HCCs

We analyzed expression profiles of 20 HCCs with the corresponding non-cancerous liver tissues using the cDNA microarray containing 23040 genes. Among commonly up-regulated

genes in HCCs, a gene with an in-house accession number of C2298, corresponding to HES6 (Hs.42949 of a UniGene cluster at http://www.ncbi. nlm.nih.gov/UniGene/), was over-expressed in eleven of twelve HCCs compared with the corresponding noncancerous liver tissues (Figure6a). To clarify the results of the microarray, we carried out semi-quantitative RT-PCR and revealed that expression of HES6 was increased in 7 out of additional 8 HCCs compared with their corresponding non-cancerous liver tissues (Figure 6b).

Identification, expression, and structure of HES6

Homology searches with the sequence of C2298 in public databases using BLAST program in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) identified cDNA sequences including GenBank accession number BC007939 that corresponded to *HES6*, and a genomic sequence with GenBank accession number of AA357675 assigned to chromosomal band 2q37. *HES6* cDNA sequence consisted of 1375 nucleotides containing an open reading frame of 675 nucleotides(SEQ ID NO: 33) encoding a putative 224-amino-acid protein(SEQ ID NO: 34) (GenBank accession number BC007939). The first ATG was flanked by a sequence (GGCATGG) that agreed with the consensus sequence for initiation of translation in eukaryotes. Comparison of HES6 cDNA and the genomic sequence disclosed that this gene consisted of 4 exons. Additionally, we carried out Multiple-Tissue northern-blot analysis with a PCR product of HES6 as a probe, and detected a 1.4 kb-transcript that was expressed in testis, spinal code and skeletal muscle (Figure 7). A search for protein motifs with the Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de) revealed that the predicted protein contained a helix-loop-helix domain and orange domain (codons 31-80, 94-135) (Figure 8).

Subcellular localization of HA-tagged HES6 protein

To investigate the subcellular localization of HES6 protein, we transiently transfected a plasmid expressing HA-tagged (pCMV-HA-HES6) into COS7 cells. Western blot analysis using extracts from the cells and anti-HA antibody revealed a 30-kDa band corresponding to the tagged protein (Figure 9a). Subsequent immunohistochemical staining of the cells with these antibodies indicated that the protein was mainly present in the nucleus (Figure 9b).

Effect of plasmids expressing HES6-siRNA on growth of hepatocellular carcinoma cells.

To investigate the function of HES6 in cancer cells, we constructed plasmids expressing

HES6-siRNA and examined their effect on HES6 expression. Transfection Alexander and HepG2 cells with psiH1BX-HES6-2, psiH1BX-HES6-3, psiH1BX-EGFP or psiH1BX-mock revealed that psiH1BX-HES6-2 significantly suppressed expression of HES6 in the cells compared to psiH1BX-HES6-3, psiH1BX-EGFP or psiH1BX-mock (Figure 10a). To test whether the suppression of HES6 may result in growth suppression of HCC cells, we transfected Alexander and HepG2 cells with psiH1BX-HES6-2, psiH1BX-HES6-3, psiH1BX-EGFP or psiH1BX-mock. Viable cells transfected with psiH1BXNo2-HES6 were markedly reduced compared to those transfected with psiH1BXNo3-HES6, psiH1BX-EGFP or psiH1BX-mock suggesting that decreased expression of HES6 suppressed growth of hepatocellular carcinoma cells (Figure 10b).

EXAMPLE3. DISCUSSION

cDNA microarray technologies have enabled us to obtain comprehensive profiles of gene expression in various human neoplasms. This approach discloses the complex nature of cancer cells, and helps for the profound understanding of carcinogenesis. In addition, it facilitates the identification of genes whose expression levels are deregulated in tumors, which should lead to more precise diagnosis of the tumors, and the development of novel therapeutic strategies.

Studies designed to reveal mechanisms of carcinogenesis have identified several molecular targets for anti-tumor agents. For example, inhibitors of farnesyltransferase (FTIs) were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends upon posttranslational farnesylation, and have been effective in treating Ras-dependent tumors in animal models (5). In humans, clinical trials using a combination of anti-cancer drugs and an anti-HER2 monoclonal antibody, trastuzumab, to antagonize the proto-oncogene receptor HER2/neu, have improved clinical response and overall survival of a subset of breast-cancer patients (6). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcl-abl fusion proteins, has been developed to treat chronic myelogenous leukemias where constitutive activation of bcr-abl tyrosine kinase plays a crucial role in transformation of leukocytes. Agents of this kind are designed to suppress oncogenic activity of specific gene products (7). From the pharmacogenetic point of view, suppressing oncogenic signals is easier in practice than activating tumor-suppressive effects. Therefore commonly up-regulated gene product such as *MGC47816* and *HES6* represents promising potential target for designing novel anti-cancer agents.

We have demonstrated here that suppressing the expression of *MGC47816* and *HES6* by

short interfering RNA (siRNA) markedly decreases growth of cancer cells. Although the precise molecular mechanism by which the short interfering RNA (siRNA) can suppress growth needs to be clarified, our data clearly indicate that this gene could be good candidate as diagnostic marker for HCCand could represent molecular target for development of effective drugs to treat this intractable tumor.

Industrial Applicability

The previous gene-expression analysis of genome-wide cDNA microarray has identified specific up-regulated gene *MGC47816* or *HES6*. The present invention revealed *MGC47816* or *HES6* serves as target for cancer prevention and therapy. Based on the expression of MGC47816 or HES6, the present invention provides a molecular diagnostic marker for identifying or detecting HCC.

The methods described herein are also useful in the identification of additional molecular targets for prevention, diagnosis and treatment of HCC. The data reported herein add to a comprehensive understanding of HCC, facilitate development of novel diagnostic strategies, and provide clues for identification of molecular targets for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of hepatocellular tumorigenesis, and provide indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of HCC.

All patents, patent applications, and publications cited herein are incorporated by reference in their entirety. Furthermore, while the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

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What is claimed is:

1. A method of diagnosing HCC or a predisposition to developing HCC in a subject, comprising determining a level of expression of MGC47816 or HES6 in a patient derived biological sample, wherein an increase of said level compared to a normal control level of said gene indicates that said subject suffers from or is at risk of developing HCC.
2. The method of claim 1, wherein said increase is at least 10% greater than said normal control level.
3. The method of claim 1, wherein the expression level is determined by any one method select from group consisting of:
   (a) detecting the mRNA of MGC47816 or HES6,
   (b) detecting the protein encoded by MGC47816 or HES6, and
   (c) detecting the biological activity of the protein encoded by MGC47816 or HES6,
4. The method of claim 3, wherein said detection is carried out on a DNA array.
5. The method of claim 1, wherein said biological sample comprises an epithelial cell.
6. The method of claim 1, wherein said biological sample comprises HCC cell.
7. The method of claim 1, wherein said biological sample comprises an epithelial cell from a HCC.
8. A method of screening for a compound for treating or preventing HCC, said method comprising the steps of:
   a) contacting a test compound with a polypeptide encoded by a nucleic acid of MGC47816 or HES6;
   b) detecting the binding activity between the polypeptide and the test compound; and
   c) selecting a compound that binds to the polypeptide.
9. A method of screening for a compound for treating or preventing HCC, said method comprising the steps of:
   a) contacting a candidate compound with a cell expressing MGC47816 or HES6, and
   b) selecting a compound that reduces the expression level of MGC47816 or HES6.
10. The method of claim 9, wherein said cell comprises a hepatocellular carcinoma cell.
11. A method of screening for a compound for treating or preventing HCC, said method comprising the steps of:
   a) contacting a test compound with a polypeptide encoded by a nucleic acid of MGC47816 or HES6;
   b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by a nucleic acid of MGC47816 or HES6 in comparison with the biological activity detected in the absence of the test compound.

12. The method of claim 11, wherein the biological activity of the polypeptide is cell proliferative activity.

13. A method of screening for compound for treating or preventing HCC, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of MGC47816 or HES6 and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene, as compared to a control.

14. A kit comprising a detection reagent which binds to nucleic acid sequence or polypeptide of MGC47816 or HES6.

15. A method of treating or preventing HCC in a subject comprising administering to said subject an antisense composition, said composition comprising a nucleotide sequence complementary to a coding sequence of MGC47816 or HES6.

16. A method of treating or preventing HCC in a subject comprising administering to said subject a siRNA composition, wherein said composition reduces the expression of a nucleic acid sequence of MGC47816 or HES6.

17. The method of claim 16, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 21 ,22, 31 or 32 as the target sequence.

18. A method for treating or preventing HCC in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by nucleic acid of MGC47816 or HES6.

19. A method of treating or preventing HCC in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid of MGC47816 or HES6 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide.

20. A method for treating or preventing HCC in a subject, said method comprising the step of administering a compound that is obtained by the method according to any one of claims 8-13.

21. A composition for treating or preventing HCC, said composition comprising a pharmaceutically effective amount of an antisense polynucleotide or small interfering RNA against a polynucleotide of MGC47816 or HES6, as an active ingredient, and a pharmaceutically acceptable carrier.
22. The composition of claim 21, wherein the siRNA comprises a sense strand comprising the nucleotide sequence of SEQ ID NO: 21, 22, 31 or 32 as the target sequence.
23. A composition for treating or preventing HCC, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by nucleic acid of MGC47816 or HES6, as an active ingredient, and a pharmaceutically acceptable carrier.
24. A composition for treating or preventing HCC, said composition comprising a pharmaceutically effective amount of the compound selected by the method of any one of claims 8-13 as an active ingredient, and a pharmaceutically acceptable carrier, as an active ingredient, and a pharmaceutically acceptable carrier.
25. The small interfering RNA, wherein the sense strand thereof comprises the nucleotide sequence of SEQ ID NO: 21, 22, 31 or 32 as the target sequence.

ABSTRACT

Objective methods for detecting and diagnosing hepatocellular carcinoma (HCC) are described herein. In one embodiment, the diagnostic method involves the determining a expression level of MGC47816 or HES6 that discriminate between HCC and normal cell. The present invention further provides methods of screening for therapeutic agents useful in the treatment of HCC, methods of treating HCC and method of vaccinating a subject against HCC.

Fig. 1

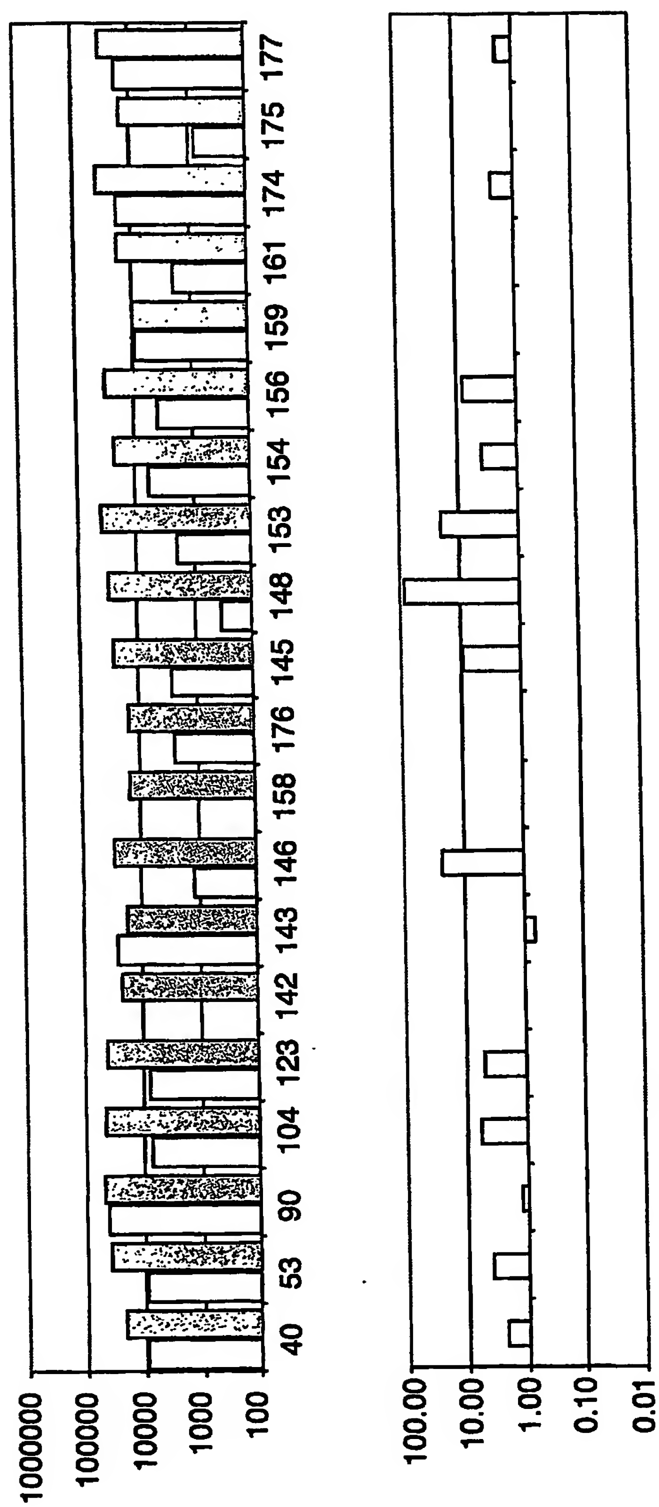
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Fig. 2

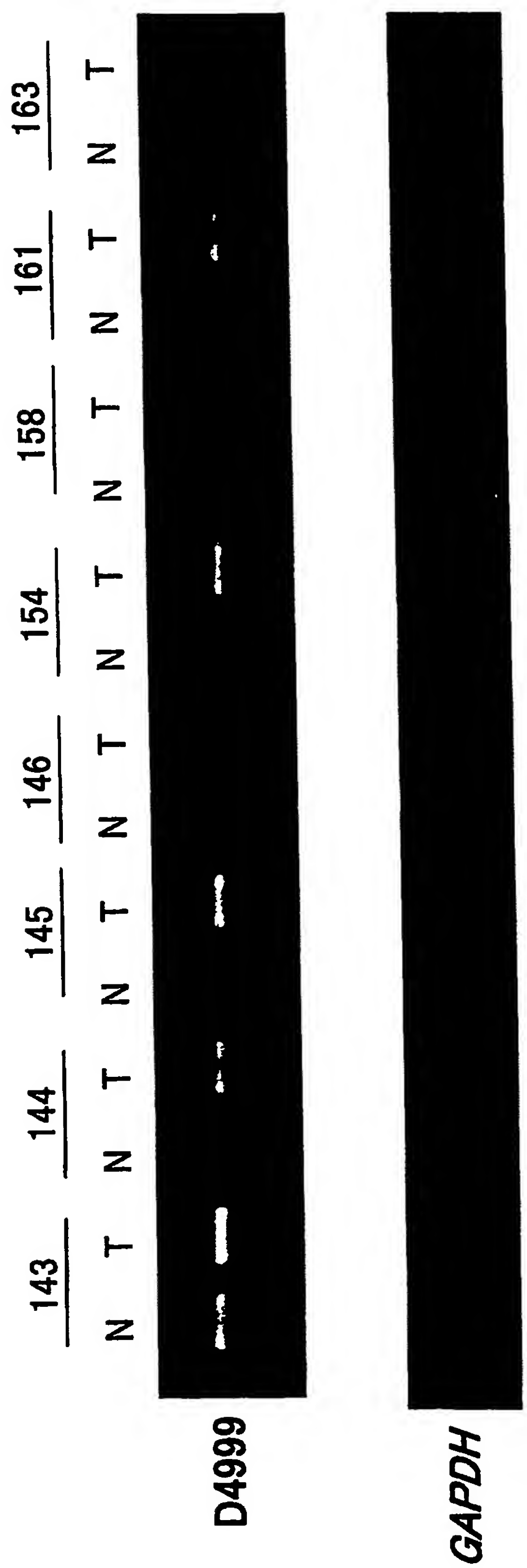
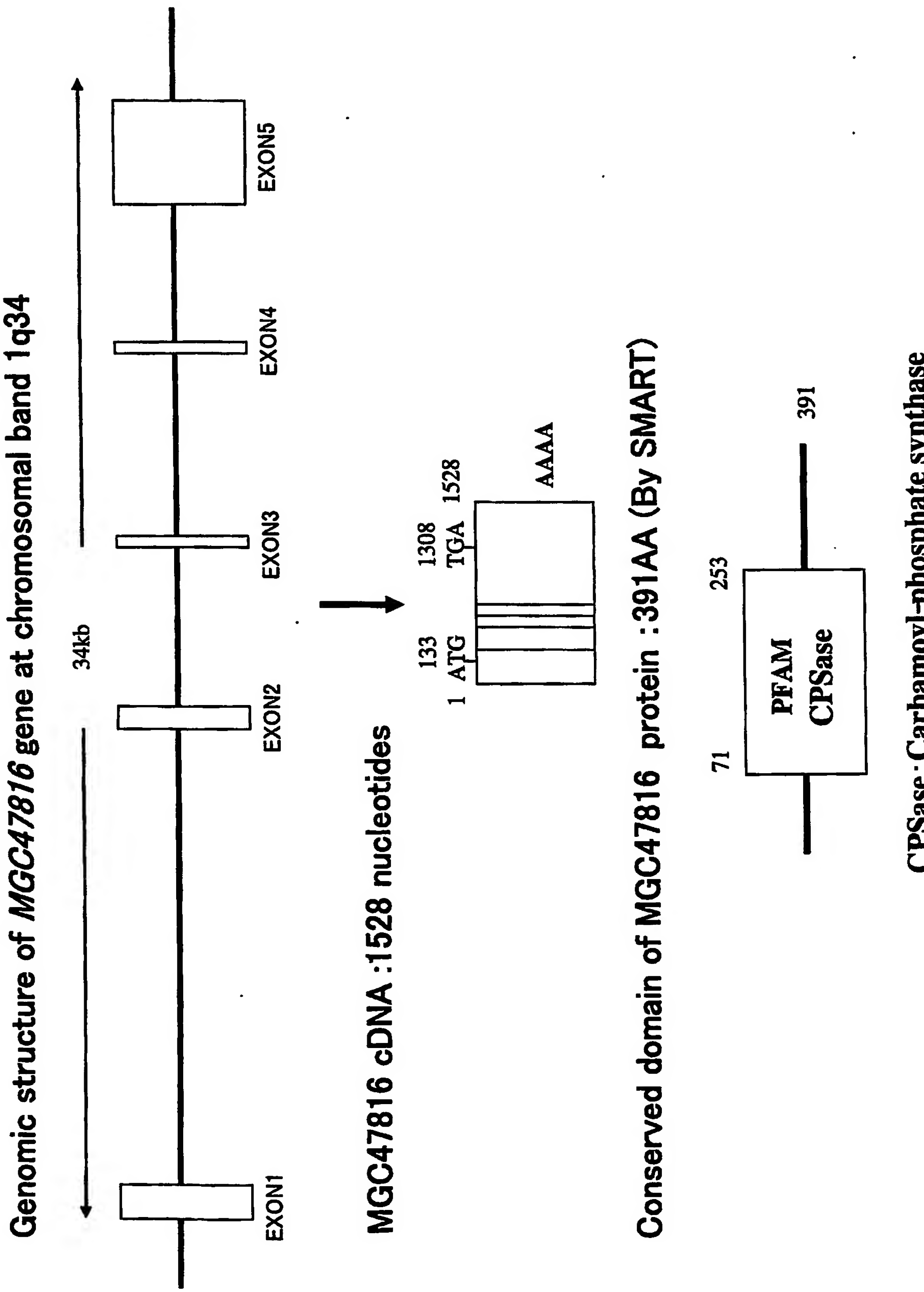


Fig. 3


Genomic structure of MGC47816 gene at chromosomal band 1q34
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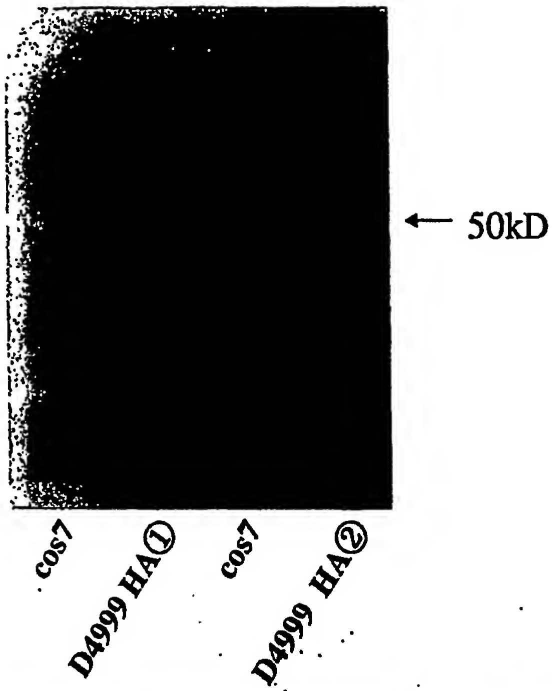
EXON1
EXON2
EXON3
EXON4
EXON5
MGC47816 cDNA :1528 nucleotides
1
133
ATG
1308
TGA
1528
AAAA
Conserved domain of MGC47816 protein :391AA (By SMART)
71
253
391
PFAM
CPSase
CPSase: Carbamoyl-phosphate synthase

Fig. 4

a



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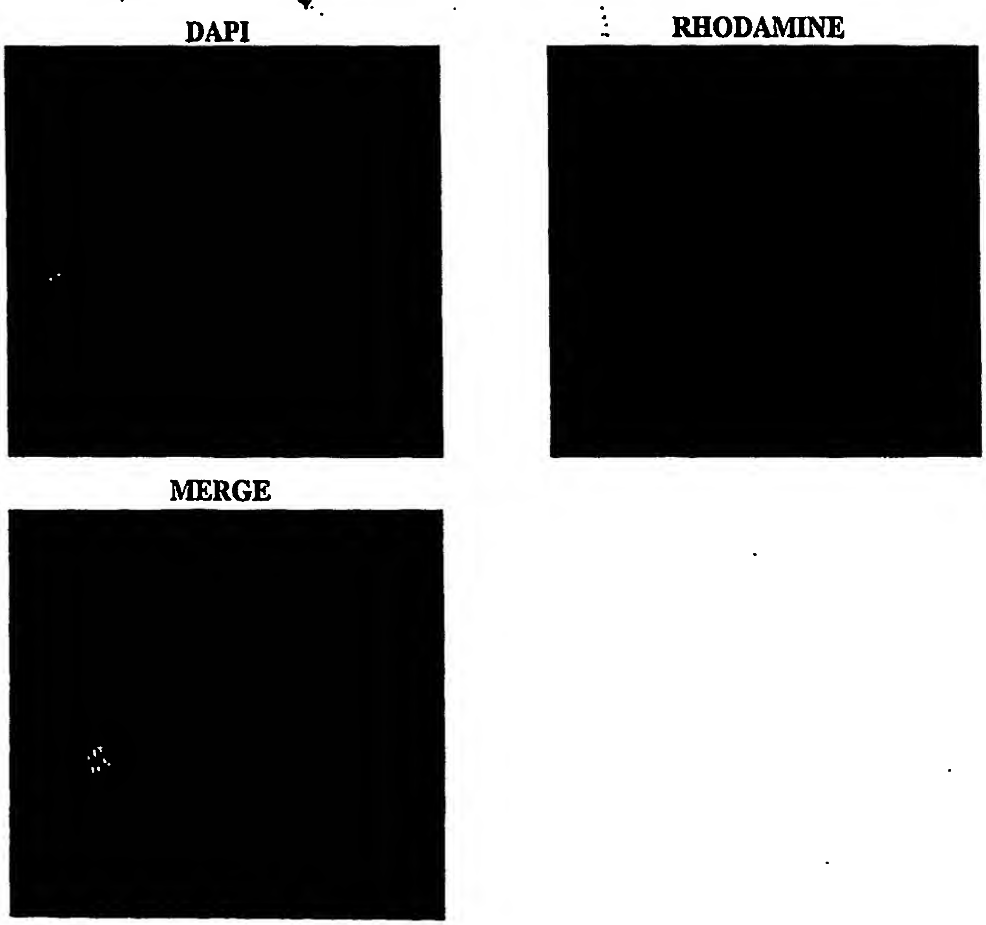
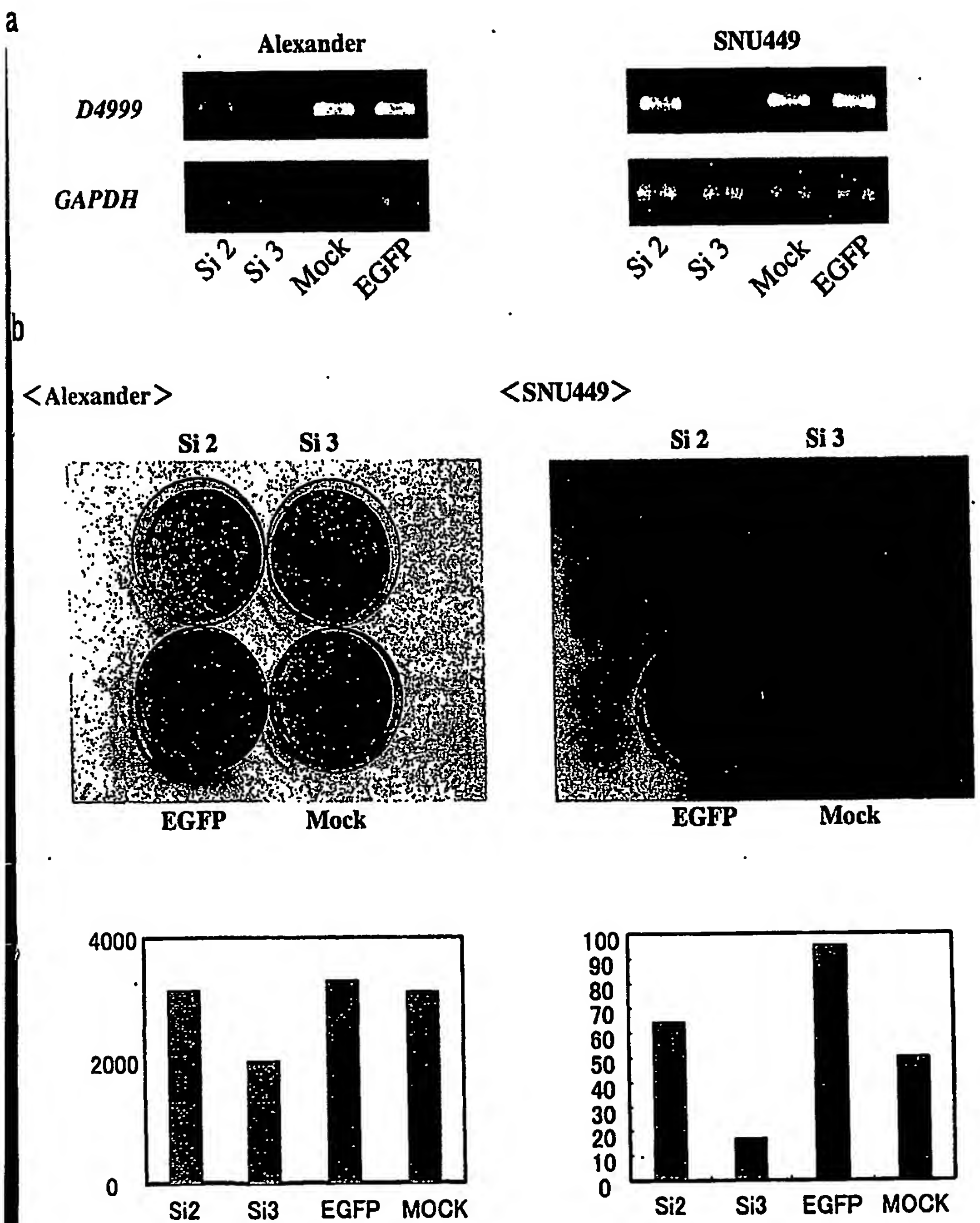


Fig. 5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GAPDH
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Si 3
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Si2
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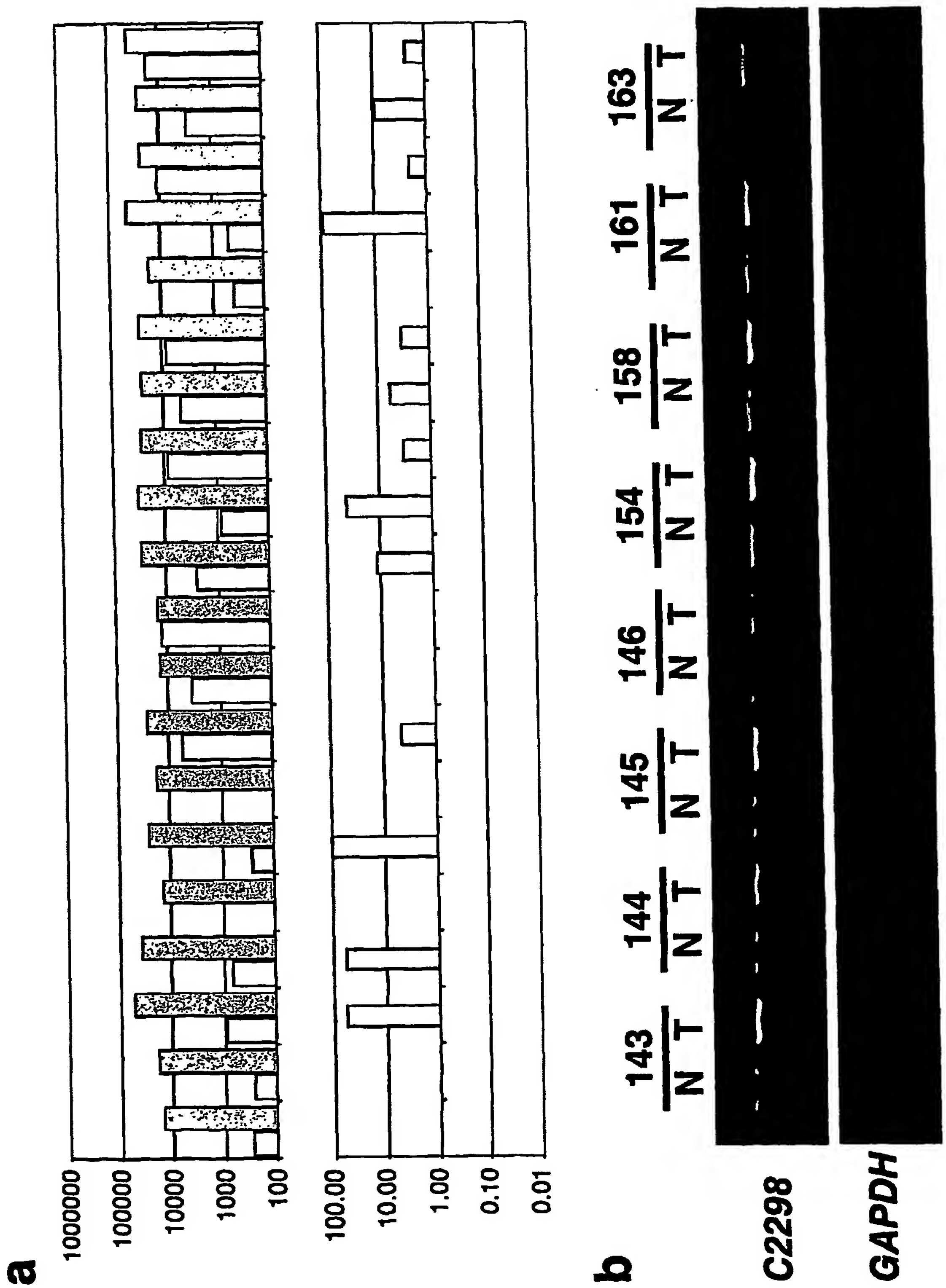
MOCK

Fig. 6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b
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N T
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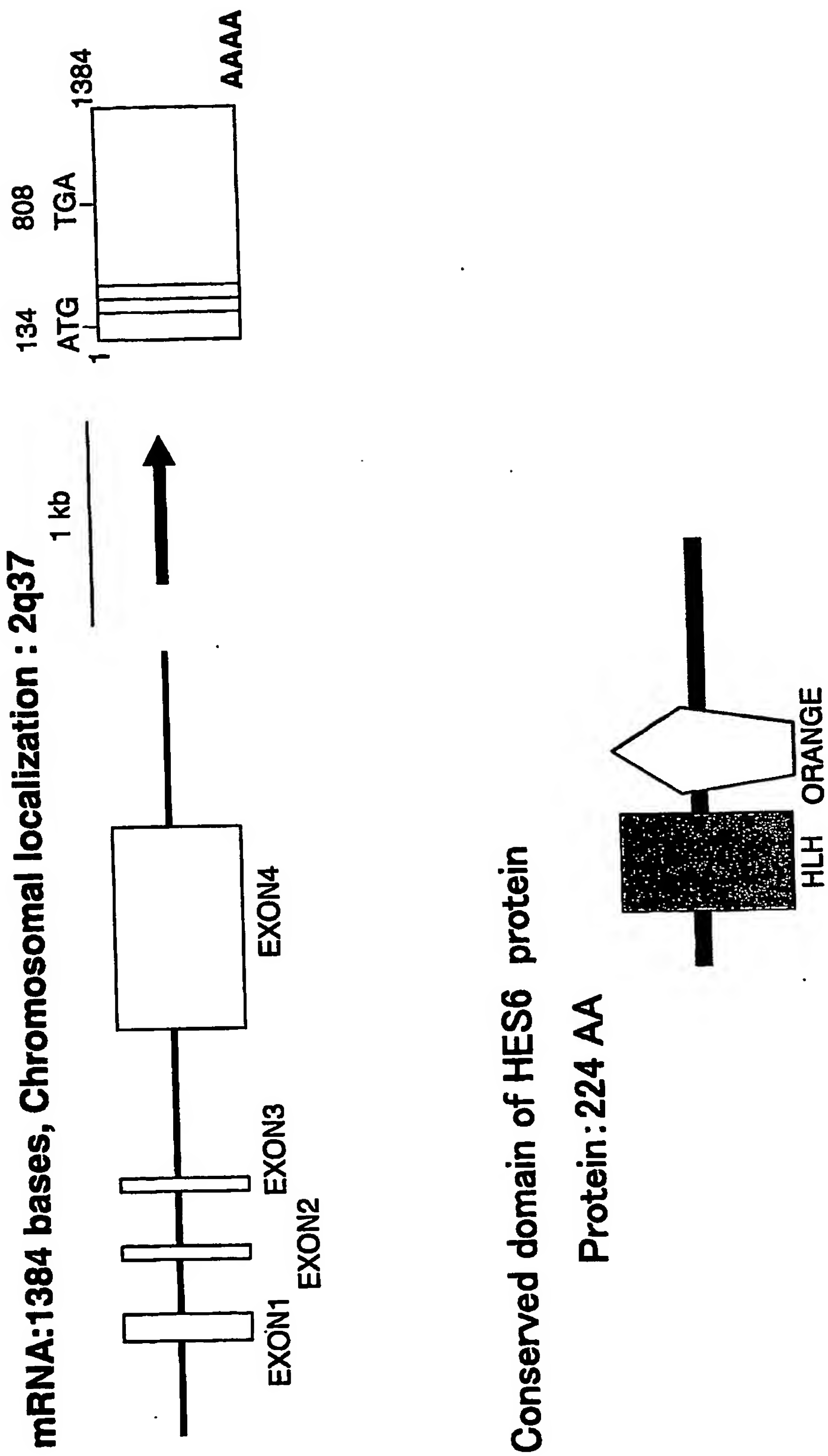
145
N T
146
N T
154
N T
158
N T
161
N T
163
N T
C2298
GAPDH

Fig. 7


HEART
BRAIN
PLACENTA
LUNG
LIVER
*SKELETAL MUSCLE
KIDNEY
PANCREAS
SPLEEN
THYMUS
PROSTATE
*TESTIS
OVARY
SMALL INTESTINE
COLON
LEUKOCYTE
STOMACH
THYROID
*SPINAL CORD
LYMPH NODE
TRACHEA
ADRENAL GLAND
BONE MARROW
1.4kb

Fig. 8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TGA
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AAAA
EXON1
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EXON3
EXON4
Conserved domain of HES6 protein
Protein:224 AA
HLH
ORANGE

Fig. 9

a


HA-tagged HES6
IB:anti-HA Ab
30kD
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pCMV-HA-HES6-1
pCMV-HA
pCMV-HA-HES6-2

b

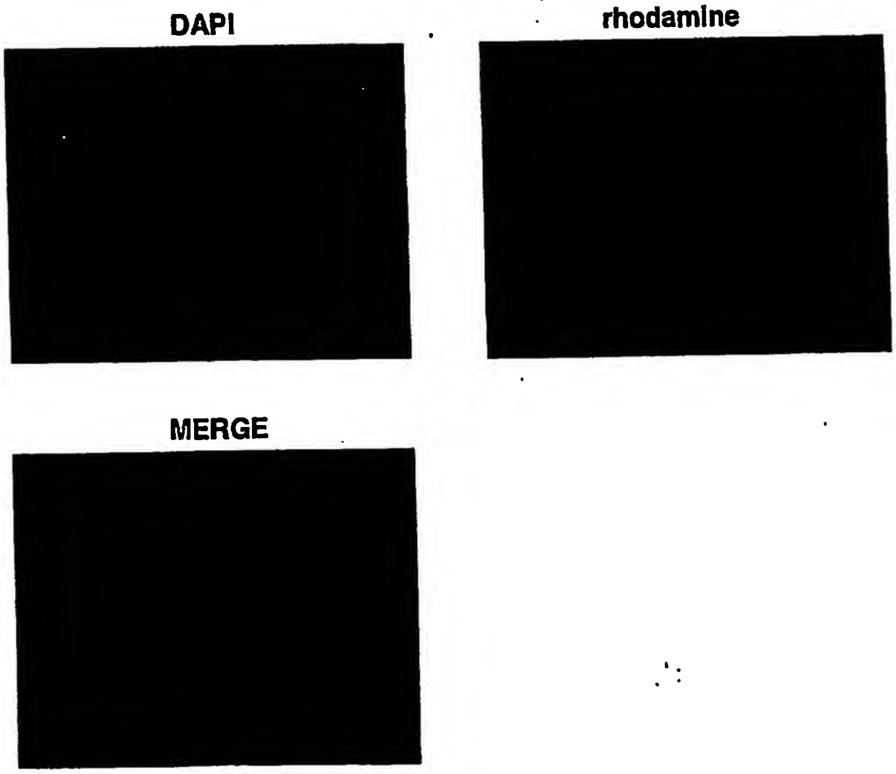
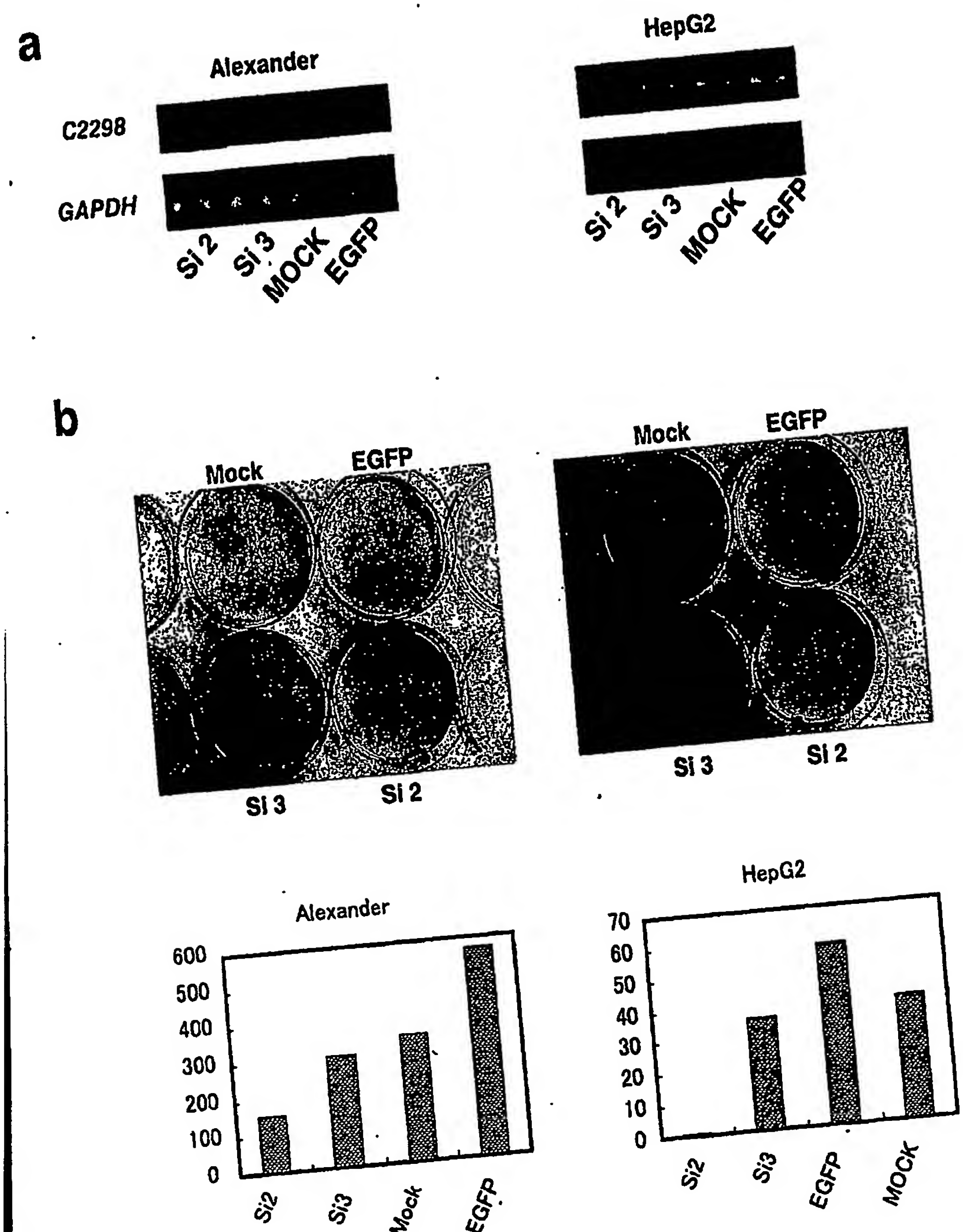

DAPI
rhodamine
MERGE

Fig. 10



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Si 3
MOCK
EGFP
HepG2
Si 2
Si 3
MOCK
EGFP
b
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Si 3
Si 2
Mock
EGFP
Si 3
Si 2
Alexander
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400
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Ser Val Gln Ser Asp Ser Asp Ile Thr Val Leu Arg His Leu Glu Lys
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tct gac atc tgc cat ctg atc cgc cac gat gtg ccc tac ctg ttc cag 699
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385 390

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Thr Phe Pro Asp Val Val Leu Val Arg Val Pro Thr Pro Ser Val Gln
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Gly Lys Ala Val Phe Leu Ala Arg Asp Lys His His Leu Ser Asp Ile
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Cys His Leu Ile Arg His Asp Val Pro Tyr Leu Phe Gln Lys Tyr Val

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Lys Glu Ser His Gly Lys Asp Ile Arg Val Val Val Val Gly Gly Gln
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Cys Gly Ile Asp Leu Leu Ile Met Asp Asp Gly Ser Phe Val Val Cys
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Gly Trp Pro Ala Gly Gly Ala Pro Gly Ser Pro Ile Pro Ser Pro Pro
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Pro Gly Asp Asp Leu Cys Ser Asp Leu Glu Glu Ala Pro Glu Ala Glu
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